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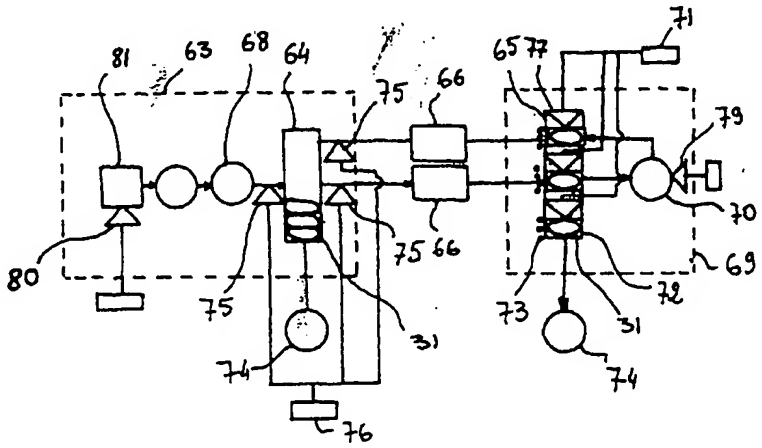
Déclarations en vertu de la règle 4.17 :

— *relative au droit du déposant de revendiquer la priorité de la demande antérieure (règle 4.17.iii)) pour la désignation suivante US*

[Suite sur la page suivante]

(54) Title: METHOD FOR DETERMINING AND MONITORING AGEING OF BLOOD BAGS IN BLOOD TRANSFUSION AND HEALTH CENTRES

(54) Titre : PROCEDE DE DETERMINATION ET DE SUIVI DU VIEILLISSEMENT DES POCHES DE SANG DANS LES ETABLISSEMENTS DE TRANSFUSION SANGUINE ET LES ETABLISSEMENTS DE SOINS



(57) Abstract: The invention concerns a method concerns a method for indicating if a bag can be delivered by transfusion to a patient or not, whereon is fixed permanently an electronic chip equipped with an antenna capable of communicating with an electronic communication device (80) and a simplified electronic communication device (75, 77) equipped with a loop antenna connected to a programmable automaton (76, 71); whereby an expiry date is defined, from an initial time determined by taking a sample in a parent blood bag in a blood transfusion centre (63) by means of the electronic communication device (80) integral with a stirring spring balance (81), said expiry date being posted in the electronic chip of a parent blood bag then transferred into the electronic chip of a primary blood bag then into the electronic chip of secondary blood bags and whereby is also defined a maximum authorized residence time dT outside a chamber with controlled atmosphere (64, 65), for qualifying anew the blood bag when it is returned to the chamber if dT and the expiry date have not elapsed and for disqualifying it if dT or the expiry date have elapsed so as to destroy it.

[Suite sur la page suivante]

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— *relative à la qualité d'inventeur (règle 4.17.iv)) pour US seulement*

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En ce qui concerne les codes à deux lettres et autres abréviations, se référer aux "Notes explicatives relatives aux codes et abréviations" figurant au début de chaque numéro ordinaire de la Gazette du PCT.

(57) Abrégé : Ce procédé indique qu'une poche peut être transfusée ou non sur un patient, sur laquelle est fixée à demeure une puce électronique équipée d'une antenne en boucle susceptible de communiquer avec un dispositif de communication électronique (80) et un dispositif de communication électronique (75, 77) simplifié équipés d'une antenne en boucle reliés à un automate programmable (76, 71), selon lequel une date limite d'utilisation est définie, à partir d'un instant initial déterminé par un prélèvement dans une poche de sang mère effectué dans un établissement de transfusion sanguine (63) grâce au dispositif de communication électronique (80) solidaire d'un peson-agitateur (81), ladite date limite d'utilisation étant inscrite dans la puce électronique d'une poche de sang mère puis transférée dans la puce électronique d'une poche de sang primaire puis dans la puce électronique de poches de sang secondaires et selon lequel est aussi définie une durée maximum de séjour autorisée dT hors d'une enceinte à atmosphère contrôlée (64, 65), permettant de requalifier la poche de sang lors de son retour dans l'enceinte si dT et la date limite d'utilisation ne sont pas dépassées et de la déqualifier si dT ou la date limite d'utilisation sont dépassés afin de la détruire.

Procédé de détermination et de suivi du vieillissement des poches de sang dans les établissements de transfusion sanguine et les établissements de soins.

L'invention concerne un procédé de détermination et de suivi du vieillissement des poches de sang dans les établissements de transfusion sanguine et les établissements de soins.

Actuellement les poches de sang sont prélevées dans les établissements de transfusion sanguine ; chaque poche est datée au moment du prélèvement ce qui marque l'origine de la vie de la poche et des produits dérivés pour une durée prédéterminée; les poches de sang prélevées subissent une filtration, une centrifugation et une séparation qui aboutit notamment à un produit dérivé qui est une poche de globules rouges destinée à être transfusée dont la durée de vie prédéterminée est de quarante cinq jours à partir du prélèvement de sang; les poches de globules rouges sont stockées dans les établissements de transfusion sanguine et délivrées au fur et à mesure des besoins des établissements de soins. Il arrive que des poches de globules rouges qui avaient été prévues pour pallier un incident en cours d'opération n'aient pas été utilisées ; comme il est impossible d'avoir actuellement une garantie certaine de la qualité du contenu de la poche de globules rouges, en vue d'une nouvelle utilisation, celle-ci est détruite. La destruction des poches de globules rouges non utilisées représentant actuellement une perte de douze pour cent, des poches de sang prélevées, qu'il est indispensable de réduire.

Les poches de globules rouges étant actuellement transportées entre l'établissement de transfusion sanguine et l'établissement de soins sans précautions particulières, une étude est en cours avec une société de transport pour garantir la continuité de la chaîne de froid entre ces deux établissements ; la poche de sang est ainsi placée dans un conteneur réfrigéré à l'intérieur duquel est placé un dispositif indiquant la température dans le conteneur tout au cours du transport; les poches de globules rouges, étant placées à l'arrivée dans un réfrigérateur approprié de l'établissement de soins, peuvent être retournées à

l'établissement de transfusion sanguine si entre temps elles n'ont pas été extraites pour être mises à la disposition d'un chirurgien.

Lors de la prise de sang, le sang est transféré dans une poche de sang mère reliée à un filtre lui-même relié à une poche primaire de sang : la poche de sang primaire est solidaire d'un groupe de trois poches secondaires auxquelles elle est reliée par des tubulures souples branchées en parallèle; le sang de la poche de sang mère est filtré et introduit dans la poche de sang primaire; la poche de sang primaire est centrifugée pour séparer les globules rouges, des plaquettes et du sérum qui sont ensuite respectivement transférés dans chacune des trois poches et c'est la poche contenant les globules rouges qui est utilisée pour réaliser les transfusions sanguines. Pour mémoire, les plaquettes ne sont isolées que lorsqu'il y a un besoin spécifique à approvisionner ; sinon elles restent avec les globules rouges et il n'y a que deux poches secondaires utilisées.

Un dispositif de traçabilité des poches de sang, selon la demande de brevet FR-9804802 est en cours de développement ; il associe une puce électronique à la poche de sang; chaque puce électronique comporte une antenne annulaire qui communique avec l'antenne annulaire d'un dispositif de communication électronique, relié à un dispositif informatique, susceptible de fournir à la puce électronique d'une part de l'énergie et d'autre part des informations qu'elle mémorise et qu'elle est susceptible de restituer audit dispositif informatique par l'intermédiaire du dispositif de communication électronique; une puce électronique mère est fixée sur la poche de sang mère qui recueille toutes les informations sur le donneur et les résultats des analyses permettant la qualification de la poche de sang mère; la puce électronique mère est fixée sur un support de puce souple de forme rectangulaire de quelques centimètres de côté sur lequel est imprimé un circuit métallisé en boucles formant l'antenne annulaire de communication; dans une version préférée de l'invention, le support de puce souple de la puce électronique primaire est placé sur une des grandes faces de la poche de sang mère et sous une étiquette rectangulaire recouvrant la plus grande

partie d'une face principale; de préférence les supports de puce mère sont toujours placés au même endroit par rapport à l'étiquette de manière à faciliter le positionnement de l'antenne du dispositif de communication électronique. Une demande de brevet FR-9908887 décrit un peson-agitateur muni d'un dispositif de communication électronique permettant d'enregistrer dans la puce électronique mère de la poche de sang mère les caractéristiques du donneur de sang et les conditions de prélèvement ; le sang de la poche de sang mère est transféré par filtration dans la poche de sang primaire; la poche de sang primaire comporte une puce électronique primaire dans laquelle sont transférées les informations contenues dans la puce électronique mère ainsi que les informations concernant les conditions de filtration; la poche de sang primaire est alors centrifugée; les constituants sont séparés et sont introduits dans les poches secondaires de sang; les poches secondaires de sang sont équipées de puces électroniques secondaires, identiques aux puces électroniques mères et primaires, qui sont fixées sur un support de puce souple placé sous une étiquette recouvrant une face de la poche secondaire de sang et de préférence à une place telle que, lorsque les poches mère, primaire et secondaire de sang sont superposées, les supports de puce électronique mère et primaire ne soient pas superposés entre eux et aux supports de puces souples secondaires; les puces électroniques secondaires, des poches secondaires, c'est-à-dire la poche de globules rouges, la poche de sérum et éventuellement la poche de plaquettes, sont renseignées par le transfert des informations de la puce électronique primaire, de la poche de sang primaire, complétées par les informations concernant les paramètres utilisés pour la séparation des composants sanguins. En ce qui concerne la poche de globules rouges ayant servi à une transfusion sanguine, la puce électronique secondaire qui lui est associée comporte en fin d'utilisation les informations concernant les conditions de son utilisation et notamment l'identité du malade transfusé.

Le procédé, objet de l'invention, consiste à formaliser tout au cours de son parcours l'état d'une poche de sang mère, primaire ou secondaire, qu'on appelle ci-après "poche

de sang", équipée d'une puce électronique, vis à vis du phénomène de biodégration qu'on qualifie ci-après de "vieillissement", de manière que l'on sache à tout moment de manière la plus précise possible si la poche de sang est qualifiée pour être transfusée.

La figure unique représente un schéma de principe des étapes du procédé de qualification, requalification, déqualification d'une poche de sang.

Actuellement, l'établissement de transfusion sanguine 63 délivre une poche de sang qualifiée pendant une période de quarante cinq jours après la date de prélèvement, dans la mesure où elle n'est pas sortie d'une enceinte à atmosphère contrôlée 64 sous le contrôle de l'établissement de transfusion sanguine 63 lui-même; la poche de sang est extraite pour un emploi immédiat dans un établissement de soins 69 et elle est systématiquement détruite si elle n'a pas été utilisée.

L'objet de l'invention consiste à permettre une requalification de la poche de sang qui est sortie de l'enceinte à atmosphère contrôlée 64; pour cela il est alors défini une durée maximum "dT" d'un séjour en dehors d'une enceinte à atmosphère contrôlée 64, 65 ; chaque fois que la poche de sang 31 fait un séjour en dehors d'une enceinte à atmosphère contrôlée 64,65, la puce électronique est interrogée grâce à un dispositif de communication électronique 75,77 simplifié équipé d'une antenne en boucle, relié à un automate programmable 76,71 ; si la durée du séjour en dehors de l'enceinte à atmosphère contrôlée est inférieure à dT, la poche de sang est dite "requalifiée" si le délai de quarante-cinq jours depuis le prélèvement n'est pas dépassé ; le délai de quarante-cinq jours permet de définir une date qu'on appelle ci-après "date limite d'utilisation" qui est inscrite dans la puce électronique de la poche de sang mère par un dispositif de communication électronique 80 solidaire d'un peson-agitateur 81 et ensuite transféré aux poches de sang primaires après filtration et secondaires après la phase de séparation 68. Dans ces conditions, lorsque la poche de sang 31 est stockée dans l'enceinte à atmosphère contrôlée 64 de l'établissement de

transfusion sanguine 63 après la phase de séparation 68, les seules informations caractérisant son vieillissement est la date limite d'utilisation et la durée maximum de séjour autorisée dT hors d'une enceinte à atmosphère contrôlée 64,65 et la date d'entrée dans l'enceinte à atmosphère contrôlée.

Lorsque la poche de sang 31 est extraite de l'enceinte à atmosphère contrôlée 64 pour être envoyée vers un établissement de soins 69 la date de sortie est comparée à la date limite d'utilisation de la poche de sang 31 et inscrite dans la puce électronique de la poche de sang 31 ; la poche de sang 31 est dite "qualifiée" si la date limite d'utilisation n'est pas dépassée ; si la date limite d'utilisation est dépassée la poche de sang 31 est dite "déqualifiée" et envoyée à la destruction. La poche de sang 31 est transportée vers l'établissement de soins 69 dans un véhicule comportant une enceinte réfrigérée 66 et la poche de sang 31 est requalifiée ou déqualifiée à l'arrivée au moment du transfert de la poche de sang 31 vers l'enceinte à atmosphère contrôlée 65 de l'établissement de soins 69, sur la base de l'enregistrement des températures de l'enceinte réfrigérée 66 en cours de transport ; si la température de transport a été respectée la poche de sang est requalifiée et la date de requalification est inscrite dans la puce électronique de la poche de sang 31 ; si la température de transport n'a pas été respectée pendant une durée inférieure ou égale à dT elle est aussi requalifiée et la date de requalification est inscrite dans la puce électronique de la poche de sang 31; si la durée dT, de non respect de la température de transport, est dépassée la poche est déqualifiée la date n'est pas inscrite et la poche de sang est envoyée à la destruction ; la poche de sang 31 requalifiée est alors introduite dans l'enceinte à atmosphère contrôlée 65 de l'établissement de soins 69.

Lors de l'extraction de la poche de sang 31 de l'enceinte à atmosphère contrôlée 65 pour l'envoyer en salle d'opération 70, la poche de sang 31 est requalifiée ; si la poche de sang 31 n'est pas utilisée et qu'elle est retournée vers l'enceinte à atmosphère contrôlée 65, elle est requalifiée et remise dans l'enceinte à atmosphère contrôlée 65 ou déqualifiée. Dans un souci de fiabilité, il peut être

utilisé au niveau de l'établissement de soins une enceinte à atmosphère contrôlée 65 cellulaire du type de celle précédemment décrite dans la demande de brevet FR 0107618 mais qui est gérée par l'automate programmable 71 au lieu d'être gérée par un ordinateur, chaque alvéole 72 étant équipée d'au moins trois lampes témoins 73 ; cet automate programmable 71 vérifie chaque alvéole 72 à intervalles de temps réguliers et rapprochés très inférieurs à dT ; si l'alvéole 72 contient une poche de sang 31, il contrôle la date limite d'utilisation, la date du dernier contrôle inscrit dans la puce électronique et allume celle des trois lampes témoin 73 qui correspond au résultat du contrôle ; il inscrit la nouvelle date de contrôle et allume une lampe témoin verte si la date limite d'utilisation est suffisamment éloignée et le temps écoulé depuis la dernière qualification ou requalification est inférieur à dT, ou il allume une lampe témoin orange si la date limite d'utilisation est proche et le temps écoulé, depuis la dernière requalification, est inférieur à dT ; il allume une lampe témoin rouge sans inscrire dans la puce électronique de la poche de sang la nouvelle date de contrôle pour indiquer que la poche est déqualifiée et doit être détruite dans un incinérateur 74, soit parce que la date limite d'utilisation est dépassée, soit parce que le temps maximum dT est dépassé ; la lampe témoin orange indique aussi, lorsqu'elle est allumée, qu'il faut renvoyer la poche de sang vers l'établissement de transfusion sanguine 63 si l'établissement de soins n'en a pas l'usage immédiat pour éviter la déqualification de la poche de sang, si le délai disponible pour atteindre la date limite d'utilisation est suffisant pour permettre le transfert vers le centre de transfusion sanguine d'une part puis vers un nouvel établissement de soins d'autre part.

Lorsque la poche de sang subit le contrôle ultime par l'intermédiaire d'un dispositif de communication électronique autonome 79 au moment de la transfusion, parmi l'ensemble des paramètres contrôlés, le temps écoulé depuis la sortie de l'atmosphère contrôlée est vérifié et comparé à dT mais ce contrôle n'est pas enregistré dans la puce, comme pour les précédents contrôles, seule la date de transfusion faisant foi.

Si dans le cadre d'un fonctionnement normal la seule détermination d'une durée maximum dT est suffisante pour garantir la qualification d'une poche de sang à être transfusée, il peut être évité des dérives du système en limitant le nombre de séjours ou le temps cumulé des séjours d'une poche de sang en dehors d'une enceinte à atmosphère contrôlée.

Revendications

1-Procédé de qualification, requalification et déqualification d'une poche de sang, indiquant que cette dernière peut être transfusée ou non sur un patient, sur laquelle est fixée à demeure une puce électronique équipée d'une antenne en boucle susceptible de communiquer avec un dispositif de communication électronique (80) et un dispositif de communication électronique (75,77) simplifié, équipés d'une antenne en boucle, reliés à un automate programmable (76,71), selon lequel une date limite d'utilisation est définie, à partir d'un instant initial déterminé par un prélèvement dans une poche de sang mère effectué dans un établissement de transfusion sanguine (63) grâce au dispositif de communication électronique (80) solidaire d'un peson-agitateur (81), ladite date limite d'utilisation étant inscrite dans la puce électronique d'une poche de sang mère, puis transférée dans la puce électronique d'une poche de sang primaire, puis dans la puce électronique de poches de sang secondaires et selon lequel est aussi définie une durée maximum de séjour autorisée dT hors d'une enceinte à atmosphère contrôlée (64,65), permettant de requalifier la poche de sang lors de son retour dans l'enceinte (64,65) si dT et la date limite d'utilisation ne sont pas dépassées et de la déqualifier si dT ou la date limite d'utilisation sont dépassés afin de la détruire.

2-Procédé selon la revendication 1, caractérisé en ce que lorsque la poche de sang est transportée dans un véhicule comportant une enceinte réfrigérée (66) depuis une enceinte à atmosphère contrôlée (64) vers une autre enceinte à atmosphère contrôlée (65), la poche de sang est requalifiée et la date de requalification est inscrite dans la puce électronique si la température de stockage l'enceinte réfrigérée (66) en cours de transport a été respectée ou si la température n'a pas été respectée pendant une durée inférieure ou égale à dT, la poche de sang étant déqualifiée et la date n'étant pas inscrite si la durée dT est dépassée.

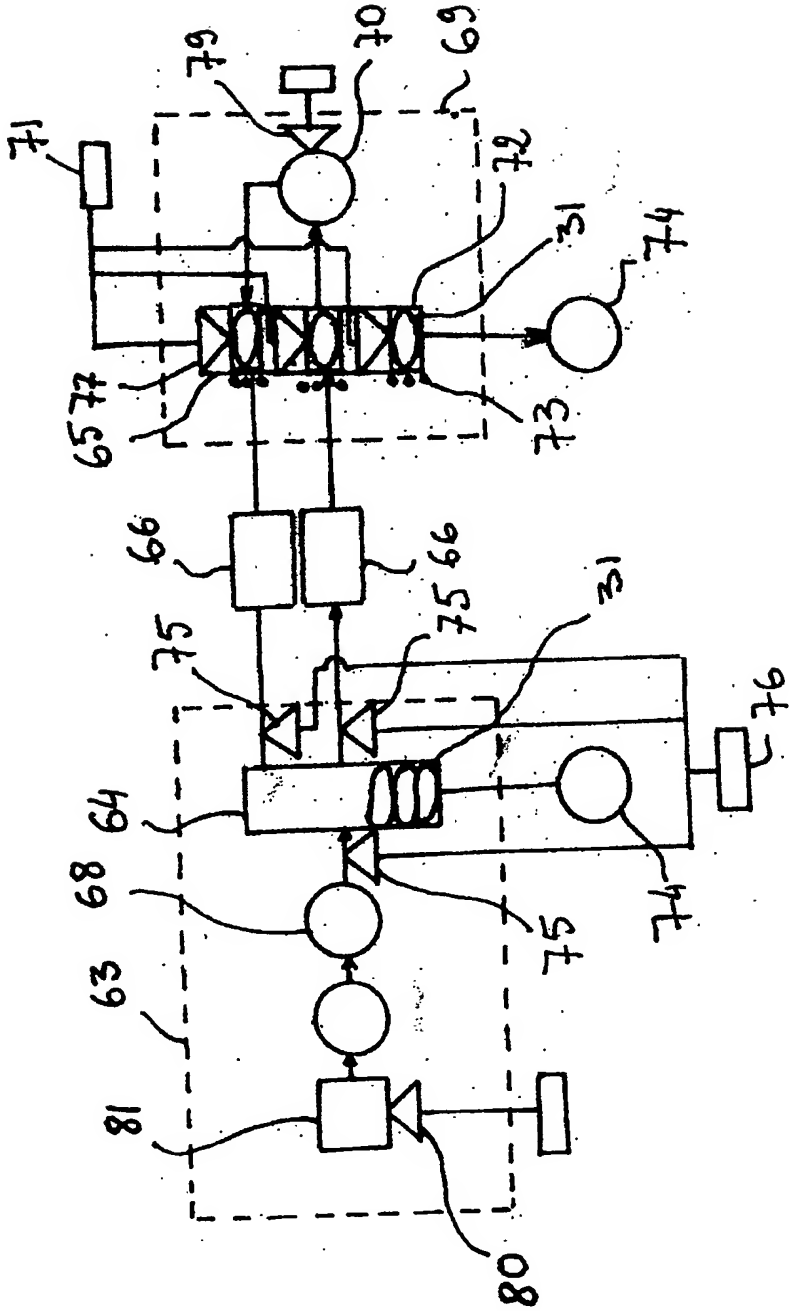
3-Procédé selon la revendication 1, caractérisé en ce que lorsque la poche de sang (31) est envoyée en salle d'opération (70) elle est requalifiée au moment de son extraction de l'enceinte à atmosphère contrôlée (65), la date de requalification étant alors inscrite dans la puce électronique et si la poche de sang (31) n'est pas utilisée elle est requalifiée, la date de requalification étant inscrite dans la puce électronique et remise dans l'enceinte à atmosphère contrôlée (65) ou déqualifiée.

4-Procédé selon la revendication 1, caractérisé en ce que la poche de sang est conservée dans une enceinte à atmosphère contrôlée (65) cellulaire dont chaque alvéole est équipée d'un dispositif de communication électronique spécialisé, l'ensemble étant géré par un automate programmable (71), les poches de sang contenues dans les alvéoles étant contrôlées à intervalle régulier en vue de les requalifier, de les déqualifier ou de détecter si la poche de sang requalifiée approche de la date limite d'utilisation, chaque alvéole étant équipée d'au moins trois lampes témoins (73) qui s'allument en fonction du résultat du contrôle.

5-Procédé suivant la revendication 1, caractérisé en ce que la poche de sang (31) subit le contrôle ultime par l'intermédiaire d'un dispositif de communication électronique autonome (79) mais ce contrôle n'est pas enregistré dans la puce électronique.

6-Procédé suivant la revendication 1, caractérisé en ce que le nombre de séjours ou le temps cumulé des séjours d'une poche de sang (31) en dehors d'une enceinte à atmosphère contrôlée (64,65) est limité.

7-Procédé suivant la revendication 1, caractérisé en ce qu'une poche de sang est renvoyée vers l'établissement de transfusion sanguine si l'établissement de soins n'en a pas l'utilisation immédiate, si le délai disponible pour atteindre la date limite d'utilisation est suffisant pour permettre le transfert vers le centre de transfusion sanguine d'une part, puis vers un nouvel établissement de soins d'autre part et que le temps écoulé depuis la dernière requalification est inférieur à dT.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AMD3100, a CxCR4 Antagonist, Attenuates Allergic Lung Inflammation and Airway Hyperreactivity

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The role of specific chemokine receptors during allergic asthmatic responses has been relatively undefined. A number of receptors are preferentially expressed on Th2 cells, including CCR4, CCR8, and CxCR4. In the present study, we have examined the role of CxCR4 in the development of cockroach allergen-induced inflammation and airway hyperreactivity in a mouse model of asthma. Using a specific inhibitor of CxCR4, AMD3100, our results indicate that blocking this receptor has a significant effect in down-regulating the inflammation and pathophysiology of the allergen-induced response. Treatment of allergic mice with AMD3100 significantly reduced airway hyperreactivity, peribronchial eosinophilia, and the overall inflammatory responses. In addition, there was a shift in the cytokine profile that was observed in the AMD3100-treated animals. Specifically, there was a significant reduction in interleukin-4 and interleukin-5 levels and a significant increase in interleukin-12 and interferon-γ levels within the lungs of treated allergic mice. Furthermore, there was a significant alteration in the local chemokine production of CCL22 (MDC) and CCL17 (TARC), two chemokines previously shown to be important in Th2-type allergen responses. Overall, specifically blocking CxCR4 using AMD3100 reduced a number of pathological parameters related to asthmatic-type inflammation. *(Am J Pathol 2002, 160:1353–1360)*

Morbidity because of asthma continues to rise, especially among children.[1–3] A significant factor in these individuals is the chronic inflammation induced by Th2-type responses that accompanies and likely precedes the most severe episodes of asthmatic disease.[4–6] Thus, considerable efforts have been made to reduce the Th2-type responses that are detrimental to the asthmatic condition. Throughout the past several years the compliant use of inhaled steroids has proven to be important in the control of atopic asthma.[7–11] However, because of continued development of steroid resistance and side effects that have been attributed to long-term steroid use, other alternatives have been explored. In particular, an attractive set of targets is the chemokine family members and their receptors that are responsible for the recruitment of particular subsets of leukocytes to the inflamed lung.[12–14] Idealistically, the targeting of the correct chemokine or receptor may have a significant impact on the development of atopic asthmatic inflammation and aid in the diminished use of steroid compounds.

Initially, the targeting of chemokines was thought to be straightforward, however, the identification of multiple chemokines and receptors have, at the very least, complicated the issue. Originally, it was perceived that the targeting of eosinophil-specific factors and their receptors, predominantly eotaxin and CCR3, might have the greatest impact on development of severe disease. However, the realization that Th subsets, Th1 and Th2, may preferentially express specific chemokine receptors suggested that these cell types could be targeted and alter the recruitment of specific T cell subsets.[15–18] The Th1 cell subset has been shown to preferentially express CxCR3 and CCR5, whereas Th2 subsets express CCR3, CCR4, CCR8, and CxCR4. Although many of these observations have come from *in vitro* analysis of cytokine-skewed responses, recent observations in animal models and in human asthma studies have begun to confirm some of these issues.[19,20] Of these Th2 cell-expressed chemokine receptors, only CxCR4, and its ligand SDF-1, have been shown to be relevant during Th2-type allergic airway responses.[21] CxCR4, like many chemokine receptors, is a Gi-coupled receptor that is specific for SDF-1.[22,23] SDF has previously been shown to be chemotactic for a number of leukocyte populations, including neutrophils, monocytes, lymphocytes, and more recently, eosinophils.[24–29] Although G-protein-coupled receptors are a pharmaceutically attractive target, few compounds have been shown to be effective during *in vivo* studies. The studies outlined in this report investigated the use of a soluble CxCR4 inhibitor, AMD3100, in the allergic lung inflammatory responses. This compound has been

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shown to be effective and specific for blocking human immunodeficiency virus entry into CxCR4-positive cells[30,31] and has further proven to be safe in phase I clinical trials.[32] In a more recent study, AMD3100 was shown to specifically inhibit SDF-1-mediated responses and alter collagen type 1 model of arthritis in mice.[33] Thus, this antagonist may provide for an effective means to block allergen-induced airway inflammation and hyperreactivity.

Materials and Methods

Animals

Female CBA/J mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and were maintained under standard pathogen-free conditions

Sensitization and Induction of the Airway Response

To induce a Th2-type response, normal mice were sensitized and challenged with cockroach allergen as previously described.[34–36] Briefly, mice were immunized with 10 μg of cockroach allergen (Bayer Corp., Elkhart, IN) in incomplete Freund's adjuvant on day 0. To localize the response to the lung the mice were given an intranasal administration of 10 μg of cockroach allergen in 10 μl of diluent on day 14. This initial intranasal challenge with antigen induced little cellular infiltrate. Mice were then challenged 6 days later by intratracheal administration of 10 μg of cockroach allergen in 50 μl of sterile.

Delivery of AMD3100

In our initial experiments a bolus injection of AMD3100 was administered intraperitoneally at various concentrations (0.1, 1, or 10 mg/kg, in 250 μl). AMD3100 was dissolved in saline and a saline control injection was administered in the control mice. AMD3100 was also used in chronic allergen models and levels needed to be maintained for ~72 hours. To ensure sufficient levels of the antagonist throughout the 72-hour experimental period, we used osmotic Alzet (Alza Corporation, Palo Alto, CA) pumps to deliver AMD3100 at a constant rate of 250 μg/kg/hour. The Alzet (Alza) pumps loaded with AMD3100 or saline were implanted into the peritoneum of allergic mice 1 hour before initial intratracheal allergen challenge.

Morphometric Analysis of Airway and Peribronchial Eosinophil Accumulation

To assess migration of eosinophils into the airway, we subjected the mice to a 1-ml bronchoalveolar lavage with phosphate-buffered saline (PBS) containing 25 mmol/L of ethylenediaminetetraacetic acid at various time points after challenge. The cells were then dispersed using a cytospin (Shandon Scientific, Runcorn, UK) and differentially stained with Wright-Giemsa stain. The cell types (mononuclear phagocytes, lymphocytes, neutrophils, and eosinophils) were expressed as a percentage based on 200 total cells counted/sample. Lung tissue was preserved with 4% paraformaldehyde at various time points after challenge. The fixed lungs were embedded in paraffin and multiple 50-μm sections were differentially stained with Wright-Giemsa for the identification of eosinophils and viewed at ×1000. The individual eosinophils were counted from 100 high-powered fields per lung at each time point using multiple step sections of lung. The eosinophils counted were only in the peribronchial region, this assured the enumeration of only those eosinophils within or immediately adjacent to an airway. The inflammation observed in this model was completely associated with the airway with little or no alveolitis.

Quantitation of Inflammatory Mediators by Specific Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of cytokine and chemokine proteins in whole-lung homogenate and from cell-free supernatants were measured by specific ELISA. The interleukin (IL)-4, IL-5, eotaxin, and IL-12/23 (p40 subunit) antibodies were purchased and pretested by the company (R & D Systems, Rochester, MN), whereas interferon (IFN)-γ, CCL7, and CCL22 were set up using previously described methodology with antibodies made by our laboratory.[19,35,37] Briefly, lung tissue was homogenized on ice using a tissue-tearor (Biospec Products, Racine, WI) for 30 seconds in 1 ml of PBS containing 0.05% Triton X-100. The resulting supernatant was isolated after centrifugation (10,000 × *g*). Flat-bottomed 96-well microtiter plates (Nunc Immunoplate I 96-F; Nunc, Roskilde, Denmark) were coated with 50 μl/well of rabbit polyclonal antibodies, specific for the cytokine/chemokine in question, for 16 hours at 4°C and then washed with PBS and 0.05% Tween 20. Nonspecific binding sites were blocked with 2% bovine serum albumin in PBS and incubated for 90 minutes at 37°C. Plates were rinsed four times with wash buffer and cell-free supernatants were added (neat and 1/10) followed by incubation for 1 hour at 37°C. Plates were washed four times, a secondary, biotinylated cytokine-specific antibody was added for 30 minutes, followed by four washes. In a final step, streptavidin-peroxidase conjugate (Bio-Rad, Richmond, CA) was added, and the plates were incubated for 30 minutes at 37°C. Plates were washed again and chromogen substrate (Bio-Rad) was added and incubated at room temperature to the desired extinction. The reaction was terminated with 50 μl/well of 3 mol/L of H_2SO_4 solution and the plates were read at 490 nm in an ELISA reader. Standards were 0.5-log dilutions of recombinant protein from 1 pg/ml to 100 ng/ml. The ELISAs with purchased reagents were sensitive to 10 pg/ml; whereas the ELISAs developed using our own reagents were sensitive to 50 pg/ml.

Measurement of Airway Hyperreactivity

Airway hyperreactivity was measured using a Buxco mouse plethysmograph, which is specifically designed

for the low tidal volumes (Buxco, Troy, NY) as previously described.[34–36,38] Briefly, the mouse to be tested was anesthetized with sodium pentobarbital and intubated via cannulation of the trachea with an 18-gauge metal tube. The mouse was subsequently ventilated with a Harvard pump ventilator (tidal volume = ~0.2 ml, frequency = 120 breaths/minute, positive end-expiratory pressure 2.0 to 2.5 cm H_2O) and the tail vein was cannulated with a 27-gauge needle for injection of the methacholine challenge. The plethysmograph was sealed and readings were monitored by computer. The trachea transducer was calibrated at a constant pressure of 20 cmH_2O. Resistance is calculated by the Buxco software by dividing the change in pressure (P_{tp}) by the change in flow (F) ($_{tp}$/F; units = cmH_2O/ml/second) at two time points from the volume curve based on a percentage of the inspiratory volume. The mouse was attached to the box and ventilated for 5 minutes before acquiring readings. Once baseline levels were stabilized and initial readings were taken, a methacholine challenge was given via the cannulated tail vein. After determining a dose-response curve (10 to 500 μg/kg), an optimal dose was chosen, 100 μg/kg of methacholine. This dose was used throughout the rest of the experiments in this study and induced little change in resistance in normal, nonallergic mice. After the methacholine challenge, the response was monitored and the peak airway resistance was recorded as a measure of airway hyperreactivity.

Flow Cytometric Analysis of Lung T Lymphocytes

Flow cytometric analysis of lymphocyte subsets was performed in dispersed lung samples from normal and cockroach allergen-challenged mice. Lung homogenate leukocyte numbers were determined by enumerating the total cell number multiplied by the percentage of total lung leukocytes (as determined by differential cell staining) or individual subsets, such as CD4 or CD8 T lymphocytes as previously described.[39] The fluorescent staining procedure was performed on ice in Dulbecco's phosphate-buffered saline (D-PBS) with 2% fetal bovine serum and 0.1% sodium azide. Total cells (1×10^6) were stained in 500 μl of buffer. Pelleted cells (5 minutes, 1400 rpm) were incubated for 30 minutes on ice with specific antibody, anti-CD4, anti-CD8, or a subclass control (Pharmingen, La Jolla, CA) directly conjugated with fluorescein isothiocyanate. After incubation an additional 2 ml of cold D-PBS was added and the cells pelleted by centrifugation (5 minutes at 1400 rpm at 4°C). The pelleted cells were washed twice with D-PBS and resuspended in 100 μl of 1% paraformaldehyde for 15 minutes. After incubation the cells were centrifuged with the addition of 2 ml of D-PBS and stored at 4°C in D-PBS containing 0.1% sodium azide until analyzed by flow cytometry. Cells were analyzed within 24 hours of staining procedure.

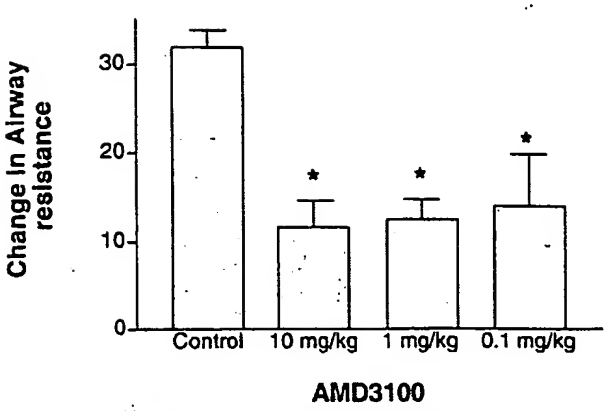

Figure 1. AMD3100 treatment significantly reduces development of AHR. Animals sensitized and challenged with cockroach allergen were given various doses of AMD3100 and assessed for development of airway hyperreactivity 24 hours after challenge. The data represents means ± SE from five to six mice per group.

Statistics

Statistical significance was determined using analysis of variance with P values <0.05.

Results

AMD3100 Treatment of Mice Reduces Allergen-Induced Airway Hyperreactivity (AHR)

Previous data has shown that blocking CxCL12 (SDF-1) can significantly alter airway responses. Thus, the inhibition of CXCR4 may have a significant effect on the induction of AHR because of its presence on Th2-type cells as well as on other cell populations involved in allergic responses. The ability of AMD3100 to block development of allergen-induced AHR was examined using various doses given intraperitoneally immediately before (within 15 minutes) a single allergen rechallenge. The data in Figure 1 indicates that AMD3100 has activity for inhibition of airway hyperreactivity over a broad range of doses. Although the effect of inhibiting airway hyperreactivity was diminished slightly at the lowest dose (0.1 mg/kg) it still demonstrated a significant decrease in airway hyperreactivity at 24 hours after allergen challenge. In separate studies a lower dose of AMD3100 (0.01 mg/kg) had no significant effect on the response (data not shown).

In additional studies we have used multiple intratracheal exposures with cockroach allergen to set up a model that induces a fully Th2-induced eosinophil-dependent airway hyperreactivity response. In these studies, we have assessed the effectiveness of AMD3100 delivered by an osmotic Alzet pump deposited into the peritoneum of the allergic mice for constant delivery. This allergen model was set up with an initial intratracheal challenge given after 21 days of the sensitization process. Forty-eight hours after the initial intratracheal allergen challenge a second intratracheal allergen rechallenge was given. This was at a time when there was a significant amount of peribronchial inflammation, including mononuclear cells and eosinophils, as previously

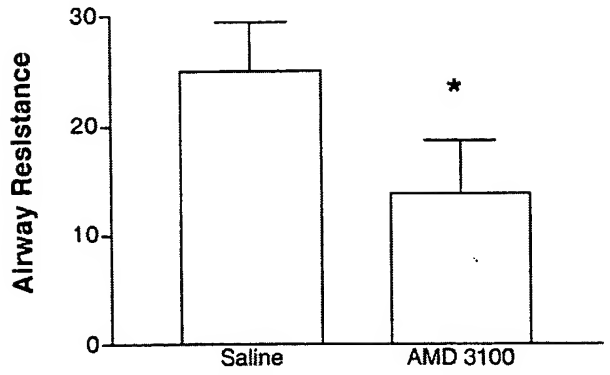

Figure 2. Inhibition of CxCR4 with AMD3100 during chronic allergic responses significantly attenuates airway hyperreactivity in allergic mice. Animals were implanted intraperitoneally with a 3-day osmotic pump that delivered 250 μg/kg/hour of AMD3100 or the saline vehicle. The cockroach allergen-sensitized mice were given two antigenic airway challenges 48 hours apart and assessed for airway hyperreactivity 24 hours after the final allergen challenge. The data represent means ± SE from six mice in each group. Control nonallergic mice treated for 3 days with Alzet pumps containing either AMD3100 or saline followed by an allergen challenge had no alteration of AHR compared to untreated control mice (airway resistance range was between 2.5 and 4.0 cmH_2O/ml/second).

described.[35] Peak airway hyperreactivity and cytokine responses occur in the lungs of sensitized mice 24 hours after the second challenge. AMD3100 was given to the mice via a 3-day Alzet pump implanted into the peritoneum 2 hours before the initial intratracheal allergen challenge. The data in Figure 2 indicates that treatment of the mice with AMD3100 (250 μg/kg/hour), via the Alzet osmotic pump, significantly reduces the severity of the airway hyperreactivity response. Thus, these results correspond to the above data using a single allergen challenge and may allow a more chronic exposure protocol to be assessed.

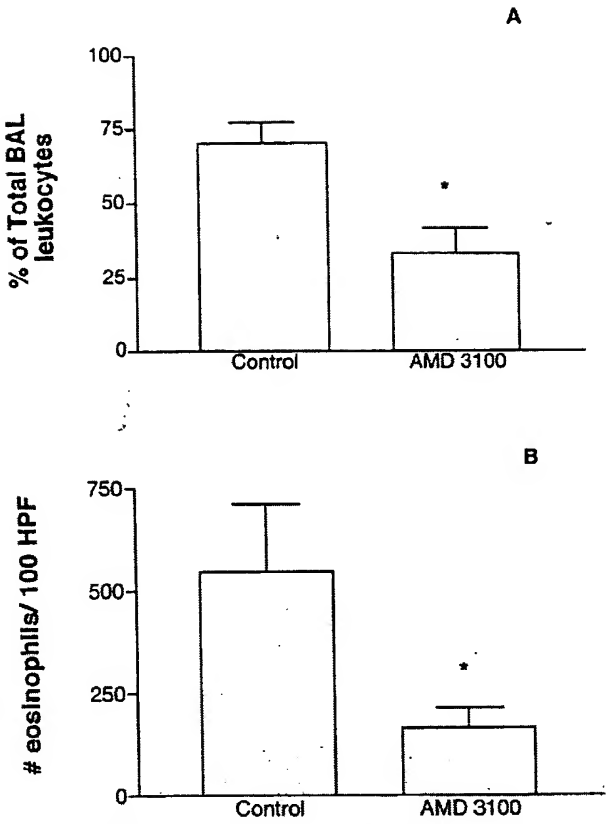

Figure 3. Allergic animals treated with AMD3100 have a significant reduction in eosinophil accumulation in and around the airway. Chronically treated allergic animals from Figure 2 were assessed for the level of eosinophil recruitment into the airway and lungs by examining the levels in bronchoalveolar lavage fluid (**A**) and enumerating the peribronchial eosinophils morphometrically (**B**). The total numbers of bronchoalveolar lavage leukocytes were 2.3 ± 1 × 10^5 for control and 1.9 ± 2.0 × 10^5 for AMD3100. The data represent the means ± SE for six mice in each group. Nonallergic control mice treated in a similar manner followed by a cockroach allergen challenge had minimal peribronchial eosinophil accumulation (<10 eosinophils/100 high-powered fields).

Analysis of Leukocyte Infiltration in Allergic Mice Treated with AMD3100

Because AMD3100 is specifically directed toward CxCR4, a chemokine receptor involved in leukocyte recruitment, we next examined whether there was an alteration in total leukocyte infiltrates and, if so, which subsets were affected? We observed a significant alteration in the accumulation of peribronchial and airway eosinophils during the chronic allergen model in the AMD3100 treatment group (Figure 3). These changes can be readily observed by histological examination of the lungs from the treated animals (Figure 4). In addition, the histology indicates an overwhelming inhibition in the overall inflammatory response. The alteration in peribronchial eosinophil accumulation and the histologically apparent alteration in the overall accumulation of cells suggested an across-the-board effect on recruitment.

To further study the nature of the altered inflammation, multiple allergen-challenged mice were implanted with Alzet pumps intraperitoneally with either saline or AMD3100 (250 μg/kg/hour). Lungs of chronic allergen-challenged mice were dispersed with collagenase and the total leukocytes were assessed along with individual subsets. Figure 5A demonstrates that AMD3100 significantly decreased the total number of leukocytes that were migrating into the lungs of chronically challenged allergic mice. We also examined whether AMD3100 altered particular T cell populations. The collagenase-dispersed lung samples from above were used for flow cytometric analysis and lymphocyte subsets were examined. The data in Figure 5B indicates that treatment of allergic mice with AMD3100 altered CD4+ cell content based on percentage of total cells that migrated into the lungs. In addition, the ratio of CD4:CD8 T cells was significantly reduced ($P < 0.05$). Together with the overall decrease in inflammation, these data strongly suggest that blocking CxCR4 with AMD3100 affects the T cell numbers and ratios of subsets and likely all subsequent responses, including eosinophil accumulation and airway hyperreactivity.

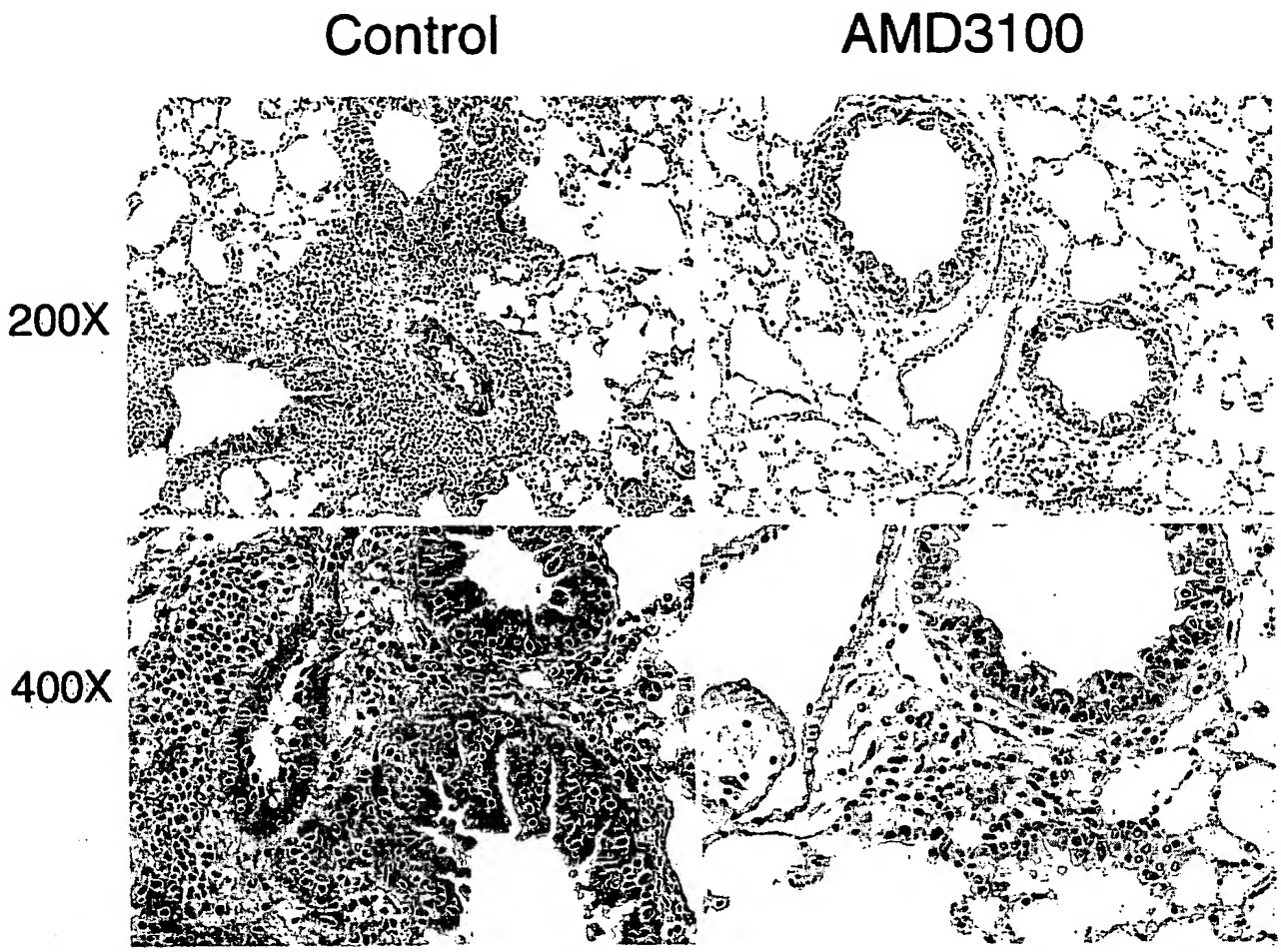

Figure 4. Inhibition of CxCR4 with AMD3100 during chronic allergic responses significantly attenuates the overall airway inflammatory responses. The photo represents the significant reduction in the leukocyte recruitment responses in the lungs of the allergic mice.

Alteration of Cytokine and Chemokine Levels after Blocking CxCR4 Interactions

In these latter studies we also assessed the local cytokine responses in the lungs of the challenged animals. The ability to reduce the appropriate cytokines (Th2 type), while increasing others (Th1 type) may be the most effective strategy to alter the long-term effects of asthmatic disease. Analysis of pulmonary levels of Th1-associated (IL-12 p40 subunit and IFN-γ) and Th2-associated (IL-4 and IL-5) cytokines indicates that AMD3100 clearly affected the cytokine profile of the lungs after multiple allergen challenges (Figure 6). Both IL-4 and IL-5 were significantly decreased, whereas IL-12 (p40 subunit) and IFN-γ were significantly increased after multiple allergen challenges in the AMD3100 treatment group. These data indicate that the phenotype of the response was drastically altered from a predominant Th2 allergic response to a more clinically attractive Th1-type response. These data help explain why there were fewer eosinophils and lower airway hyperreactivity responses.

In addition to examining Th-1 and Th2-associated cytokines, we were also interested in assessing chemokines that may be involved in the allergen-induced inflammatory responses. We were especially interested in some of those chemokines that had previously been shown to be associated with the chronic immune responses. The data in Figure 7 illustrates that when we examined CCL11 (CCR3 ligand), CCL17, and CCL22 (CCR4 ligands), both CCL17 and CCL22 were significantly reduced in the AMD3100-treated group. These data indicated that along with the Th2-type cytokines, chemokines were also significantly reduced, correlating with the overall reduction of inflammation and airway hyperreactivity.

Discussion

The rationale behind using chemokine receptor antagonists in inflammatory diseases is to selectively alter the recruitment of particular subsets of cells that initiate and maintain the inflammatory responses. In these studies, AMD3100 (a specific CxCR4 antagonist) was examined in a cockroach allergen-induced animal model of asthma. Several lines of evidence support the idea that CxCR4 may play a role in this Th2-type immune response. Possibly the most interesting evidence to date indicates that IL-4 up-regulates, whereas IFN-γ down-regulates CxCR4

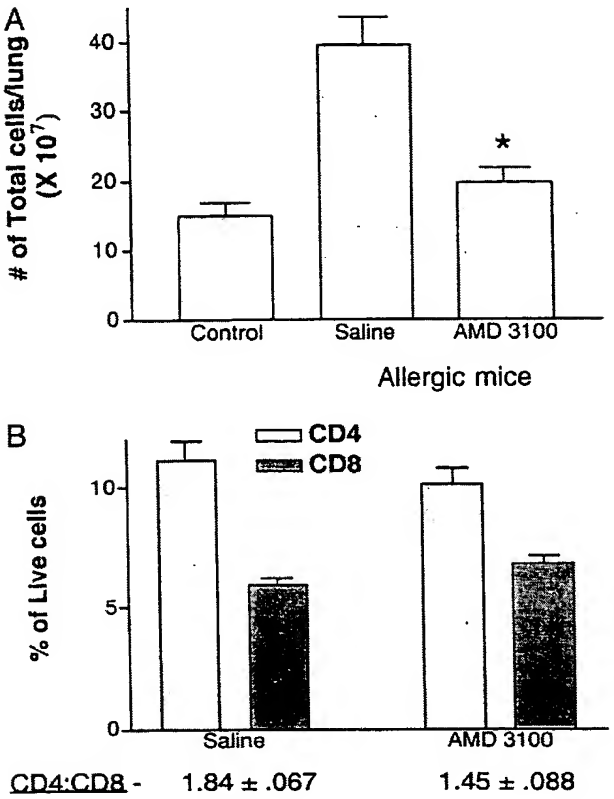

Figure 5. Blocking CxCR4 significantly reduced the accumulation of total leukocytes in the lungs of allergic mice (**A**) and significantly altered the level of CD4 + cell recruitment (**B**). Lungs from chronically allergen-challenged mice treated with AMD3100 (250 μg/kg/hour) or saline were dispersed by collagenase treatment and the single-cell suspensions assessed for leukocyte numbers and subset analysis by flow cytometry. Data represents means ± SE from four mice in each group.

expression on the surface of T lymphocytes.[18,40–42] In further support are studies indicating that Th1-type lymphocytes have very little or no expression of CxCR4, whereas Th2-type lymphocytes express CxCR4 on their surface.[20,41] In agreement with our data is a recent study that indicates that SDF-1 and CxCR4 are directly involved in the asthmatic response in an ovalbumin model of asthma.[21] Thus, the role of CxCR4 during an allergic/asthmatic pulmonary response may reflect the altered immune environment leading to increased lung damage and airway reactivity. The present experiments add further information to the possible mechanism of how CxCR4 is involved. When AMD3100 was administered to the allergic mice Th1-type cytokines were increased in the lung after allergen rechallenge. These data correlate well with the inflammation analysis, including decreased peribronchial eosinophil accumulation and the reduction in total leukocyte accumulation in allergen-challenged animals, as assessed by lung dispersion.

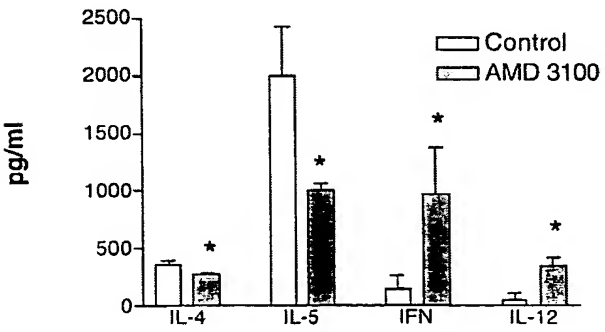

Figure 6. Alteration of cytokine profiles in allergic animals treated with AMD3100. The Th1- and Th2-associated cytokines, IL-12 (p40), IFN-γ (Th1 type), and IL-4 and IL-5 (Th2 type) were assayed in whole-lung homogenates of chronically challenged allergic mice by specific ELISA. The data represents means ± SE from five mice in each group. *, $P < 0.05$.

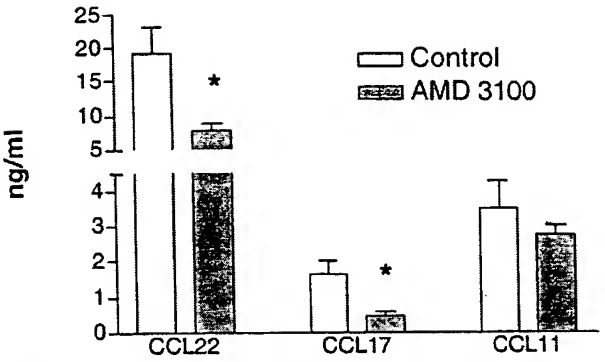

Figure 7. Reduction of chemokine production in the lungs of allergic mice treated with AMD3100. CCL11, CCL17, and CCL22 levels were assessed in the lung homogenates from mice treated with either AMD3100 (250 μg/kg/hour) or saline using specific ELISAs. The data represents means ± SE from five mice in each group. *, $P < 0.05$.

The efficacy of AMD3100 was initially tested by measuring the change in airway resistance in mice after a single allergen challenge. AMD3100 exhibited a broad dose-dependent reduction in airway resistance with significant activity as low as 0.1 mg/kg. Our subsequent studies were focused on the effect of AMD3100 during multiple allergen challenges, which may be more representative of the responses in chronic asthmatics that constantly are exposed to allergen stimulation. Because we were concerned with the availability of AMD3100 during chronic allergen exposure, we also examined the efficacy of giving AMD3100 to animals via osmotic pumps, thus allowing a constant release instead of a bolus administration. These studies demonstrated significant decreases in the airway responses and a striking alteration in the development of the peribronchial lung inflammation. Of greatest concern in asthmatics are the long-term consequences of allergen-induced inflammation and damage to the airway.[20,43] Possibly reflecting the responses in chronic asthmatics, our exposure of animals to multiple allergen challenges has previously indicated an extreme dependency on lymphocyte and eosinophil accumulation and activation.[34,35,39] The constant inflammation associated with this response may be the most devastating long-term problem to these patients. Managing this latter aspect may be the key to altering the progression of the asthmatic disease. Using AMD3100, the observed effects were broad-based and demonstrated several favorable aspects of the response. Firstly, and probably most importantly, inhibiting CxCR4 with AMD3100 significantly reduced the airway hyperreactivity response by nearly 50%. Subsequent experiments indicated an across the board reduction in inflam-

mation, including a significant reduction in peribronchial and airway eosinophilia and an alteration in lymphocyte and mononuclear phagocyte numbers. In fact, the analysis of total leukocytes indicated that there was an ~80% reduction in recruited leukocytes over background numbers in AMD3100-treated animals compared to control saline-treated animals. This effect may be because of blocking CxCR4 interactions on multiple cell populations, including lymphocytes and eosinophils, both of which have been described to express CxCR4.[29,44] In addition, recent studies have identified CxCR4 on basophils, and its ligand, SDF-1, was able to induce activation and degranulation of those cells.[45] However, the data from the present studies may build the strongest case for alteration of T cell accumulation and activation, based on the alteration of the cytokine profiles. Previous studies have clearly indicated that administration of IL-12 and/or IFN-γ in the lungs attenuates airway hyperreactivity induced by allergens.[46–48] The results from these studies not only demonstrated that IL-12 (p40 subunit) and IFN-γ levels were increased, but the administration of the CxCR4 antagonist, AMD3100, reduced IL-4 and IL-5 levels. This shift toward Th1-type cytokines may be the most favorable response because previous studies have demonstrated that Th2 cell transfer increases airway hyperreactivity, whereas transfer of Th1-type cells does not increase or alter airway hyperreactivity even though a significant response to allergen is observed.[5,49] Thus, the Th1-type cytokines, although possibly damaging, do not promote an asthmatic-type response on their own. Altogether, the alteration of cytokine profiles constitute a significant aspect of this compound that throughout time could reduce the airway damage and inflammation in patients that potentially leads to end-stage disease.

The role of chemokines in asthma is central to the chronic recruitment of leukocytes that migrate into and around the airway during asthmatic responses. A number of chemokines have been identified as possible targets using animal models of asthma and hyperreactivity, including CCL2 (MCP-1), CCL7 (MCP-3), CCL11 (eotaxin), CCL17 (TARC), CCL22 (MDC), and CxCL12 (SDF-1).[14,34,35,39,50–52] The reduction of CCL17 and CCL22 after blocking CxCR4 may represent an additional aspect of the attenuated response. The reduction of these chemokine mediators may be a direct result of the alteration of the T cell-derived cytokines, Th1 *versus* Th2. A number of chemokine receptors have also been shown to be important in the development of the airway responses, including CCR1, CCR2, and CCR8.[53–55] Relevant data for blocking other receptors, such as CCR3 and CCR4, are still lacking. However, it is likely that multiple receptors contribute to the recruitment of the various cell populations that migrate into the airways after allergen challenge. It seems that CxCR4 may be a pivotal receptor used during the allergen-induced responses. The anti-inflammatory response observed with AMD3100 treatment has been observed in a previous publication using a type I collagen-induced arthritis model,[33] but no alteration in specific subsets was seen. The present study seems to demonstrate an altered immune environment that may be because of an overall decrease in inflammation as observed in the arthritis model. In addition, CxCR4 has a significant role in leukocyte maturation in the bone marrow.[56] Thus, further studies will need to more thoroughly address whether AMD3100 has an effect on leukocyte differentiation in the bone marrow.

The specific CxCR4 inhibitor used in these studies, AMD3100, has been shown to specifically inhibit human immunodeficiency virus entry into cells via blocking CxCR4 interactions.[30,31] The pharmacokinetics and safety have already been assessed in humans and AMD3100 was found to be well tolerated.[32] There are a number of observations from the present studies that may be advantageous in other diseases as well, including the skewing of the immune response from Th2- toward a Th1-type response. Altogether, these data indicate that AMD3100 may be a desirable compound to pursue in clinical trials for efficacy in asthmatic patient populations.

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